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Cell cycle: **Flies teach an old dogma new tricks**

Pelin Cayirlioglu* and Robert J. Duronio*†‡§

E2F transcription factors are thought to influence the G1–S cell-cycle transition by controlling expression of genes required for growth and DNA synthesis. But emerging evidence suggests E2F complexes can control the cell cycle independently of transcription by directly regulating DNA replication origin usage during S phase.

Addresses: *Department of Biology, †Lineberger Comprehensive Cancer Center, ‡Program in Molecular Biology and Biotechnology and §Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599, USA.
E-mail: duronio@med.unc.edu

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Numerous changes in the profile of transcribed genes occur during progression through the eukaryotic cell cycle. Many genes are coordinately expressed at only one phase of the cell cycle, and these generally encode factors that are involved in executing the events of that particular cell-cycle phase. Consequently, these transcriptional programs are thought to contribute in a major way to progression through the cell cycle. One of the best known examples of such a program occurs at the G1–S transition, the primary proliferative control point for many cells. Work over the past 15 years on animal systems has pointed to the E2F family of transcription factors as key regulators of entry into S phase. The most widely accepted model suggests that E2Fs work exclusively by transcriptional regulation. But recent studies with both *Drosophila* and cultured mammalian cells hint at a direct involvement of E2F complexes in regulating origins of DNA replication, which is distinct from E2F's ability to regulate transcription. Here we highlight this novel function for E2F, and discuss ways in which E2F-containing complexes could directly regulate replication origin usage.

The protein dubbed 'E2F' is a heterodimeric transcription factor containing a molecule of E2F bound to a molecule of DP. In mammals, there are families of E2F and DP proteins that have both specialized and redundant functions. E2F–DP heterodimers are regulated mainly by association with a member of the pRB family of tumor suppressor proteins — pRB, p107 and p130 — each of which also has both specialized and redundant functions. In spite of the biological complexity generated by these gene families, a unifying model of how these complexes control the cell cycle has emerged. In the simplest version of the model, E2F is both an activator and repressor of transcription. Hypophosphorylated pRB binds to E2F in quiescent cells, and this complex actively represses genes containing E2F binding sites. These target genes fall into two categories: first, those that encode the 'nuts and bolts' required for DNA synthesis to initiate and proceed, such as ribonucleotide reductase, DNA polymerase α, the origin recognition complex (ORC) and so on; and second, those that encode direct regulators of cell-cycle transitions, such as cyclin E, cyclin A and the cyclin-dependent kinases (Cdks). When quiescent cells are stimulated to divide by exposure to growth factors, pRB is hyperphosphorylated by newly activated G1 cyclin-Cdk dimers and as a result dissociates from E2F. This converts E2F from a transcriptional repressor to a transcriptional activator, which induces expression of both classes of target genes and thereby stimulates entry into S phase.

This model is derived in large part from studies on mammalian cells in culture, which are tremendously useful for probing the biochemical and cellular activities of individual molecules. But because this work often relies on experiments testing the effects of overexpressing or ectopically expressing some component, the connection to normal events in intact animals is not always entirely clear. Genetic studies in whole animals can bridge this gap. Knockout mutations for each of the three pRB family members, and for four of the six E2F family members, have been generated in mice. Analyses of the phenotypes caused by these mutations have supported the general model of E2F function, and have also revealed that particular aspects of E2F function are carried out by individual E2F proteins.

The E2F-as-activator role is supported by the effects of E2F-3 deficiency: mouse embryonic fibroblasts lacking E2F-3 have a severe proliferative defect in culture [1]. Moreover, the magnitude of the growth defect in individual knockout cell lines correlates with the magnitude of loss of E2F target gene expression. Genetic experiments also support the E2F-as-repressor role. Mouse embryonic fibroblasts lacking both E2F-4 and E2F-5 fail to growth arrest in response to $p16^{INK4A}$, a cyclinD–Cdk4 inhibitor that keeps pRB in the active, hypophosphorylated state [2]. Correspondingly, simultaneous loss of all three pRB family members in cultured mouse embryonic fibroblasts results in a shortened cell doubling time, causes a failure to arrest the cell cycle in response to DNA damage, cellular contact or serum starvation, and prevents cellular senescence [3,4].

While these experiments provide important information about how pRB–E2F complexes contribute to mammalian

development, cell-cycle control and tumorigenesis, out of necessity much of the mechanistic data still come from analyses of cultured cells. This is because many of these mutations prevent normal development, and detailed cell biological analysis *in situ* is not easy in mutant mouse embryos. *Drosophila* provides distinct advantages in this regard, where it is much easier to look at cellular phenotypes in intact tissues. The pRB and E2F gene families are conserved in flies, which have two members of each family. In addition, the initial genetic data on *Drosophila* E2F fit the current dogma [5]: mutation of *dE2F* or *dDP* blocks expression of replication genes and compromises cell proliferation, and loss of *RBF* function in embryos causes transcriptional de-repression of E2F target genes and escape from G1 arrest. In several situations, however, *dE2F* mutant cells still replicate even when target gene expression is absent or very low [5,6]. This challenges the simplest version of the existing dogma, and raises the question of whether there are additional ways that pRB–E2F complexes affect DNA replication.

Just such a novel role for pRB–E2F was found by taking advantage of the opportunity *Drosophila* provides to examine the cell-cycle plasticity that occurs in all animal development. Such plasticity arises from variation of what we often consider a 'normal' cell cycle. For instance, early embryonic divisions lack gap phases altogether, and G1 regulation does not appear until 8 hours of embryogenesis. Thus, transcriptional regulation of the G1–S transition by E2F is irrelevant in early fly development. Other cell-cycle variations bypass what we often consider important control mechanisms. Many cell types naturally become polyploid during both insect and mammalian development, and in *Drosophila* this is a particularly widespread phenomenon. Polyploidization occurs through a specialized endoreduplication cycle in which there is no mitosis. Instead, the cell alternates between G and S phase, resulting in polyploidy. Such cells thus bypass the mechanisms that usually prevent re-replication and ensure the 'once and only once' replication origin firing typical of a mitotic cycle.

Fundamental cell-cycle regulatory mechanisms are often revealed by studying these seemingly unusual cell cycles. In fact, the new role for pRB–E2F complexes became apparent by examining a specialized cell cycle in the *Drosophila* ovary. A *Drosophila* egg chamber contains a cyst of 16 germ cells, one of which becomes the oocyte while the remaining 15 become support cells. This germ cell cyst is enclosed by an epithelium consisting of a single layer of somatic follicle cells. The follicle cells execute a unique cell-cycle program that provides an animal model for exploring the mechanism of initiation of DNA replication [7]. Follicle cells proliferate mitotically during formation of the egg chamber. They then endoreduplicate their DNA for four cycles, thus becoming polyploid. These polyploid follicle cells then synchronously and selectively amplify approximately 80-fold two chorion gene clusters that encode egg shell proteins. Chorion gene amplification provides the biosynthetic capacity necessary for rapid production of the egg shell.

Many of the molecules and mechanisms that control genomic replication during a typical S phase also control chorion gene amplification [7]. The amplification occurs by repeated re-initiation of an origin of replication within the chorion gene cluster. While specific initiation sites in animals have been hard to identify and so are in general poorly defined, the chorion loci provide an exception. A DNA fragment containing *cis*-acting elements sufficient to support chorion gene amplification has been defined [8,9]. These elements bind to the ORC, a six subunit complex necessary for the ordered recruitment of other *trans*-acting factors needed for initiation, such as Cdc6, the MCM helicase complex and Cdc45. This pre-initiation complex is activated in a still mysterious fashion by kinases such as cyclin E–Cdk2 and Cdc7–Dbf4 [10].

Interestingly, pRB–E2F complexes are also required for normal chorion gene amplification. While null alleles of *dE2F*, *dDP* and *RBF* are lethal, weak alleles of these three genes allow development and produce viable adult flies. The mutant female flies are sterile, however, because of defects in the follicle cells. The *dE2F* and *dDP* mutants have decreased chorion gene amplification and produce eggs with a thin shell [11]. Conversely, *RBF* mutant follicle cells over-amplify the chorion genes [12]. How does E2F affect chorion gene amplification? Curiously, even though it is well established in both flies and mammals that E2F can control the transcription of genes encoding ORC, MCM and Cdc6, the amplification defects occur without obvious changes to the expression of E2F target genes in the egg chambers. One possibility is that pRB–E2F directly regulates some aspect of origin function. Indeed, the ORC subunits Orc1 and Orc2 immunoprecipitate with dE2F and RBF in ovary extracts, and chromatin immunoprecipitations show that this complex is present at the chorion locus *in vivo* [12].

One attractive feature of follicle cells is that the localized replication at the chorion loci can be visualized *in situ* by immunostaining [13]. Orc1, Orc2, Orc5 and Cdc45 are each detected throughout the entire follicle cell nucleus during genomic replication in endoreduplication cycles. In contrast, these molecules localize specifically to the chorion clusters during amplification, consistent with their roles in initiation and progression of replication [11,14,15]. A mutant form of dE2F predicted to bind DNA poorly is associated with a loss of ORC localization to amplification foci [11], suggesting that an E2F-containing complex might facilitate recruitment of ORC to the chorion locus. Conversely, an allele of *dE2F* encoding a truncated protein lacking the pRB-binding domain increases the

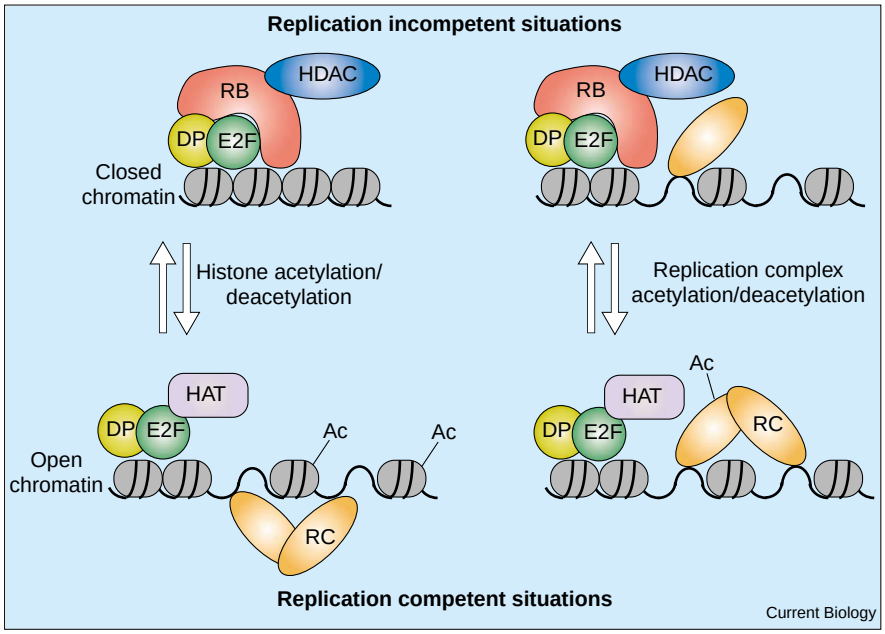
level of amplification, without a detectable change in ORC localization [11]. The truncated dE2F protein is unable to bind to the ORC, even though the complex is present at the origins in this mutant, pointing to RBF as a mediator of the E2F–ORC interaction. These data suggest that RBF–dE2F directly inhibits initiation at a step after ORC binds DNA.

Are these phenomena simply an idiosyncrasy of *Drosophila*? In a detailed look at replication in primary mammalian cells by *in situ* immunofluorescence, Kennedy *et al.* [16] found that pRB, p107 and p130 were enriched at localized sites of DNA replication early in S phase. In addition, expression of a constitutively active form of pRB, which lacks the inactivating phosphorylation sites, was found to inhibit progression through S phase in cultured cells independently of effects on G1 control [17]. These data suggest that mammalian pRB–E2F complexes may also regulate S phase independently of transcription.

What mechanism could E2F complexes use to directly regulate replication initiation? Potential answers to this question may be found by considering the enzymatic activities associated with E2F complexes. E2F associates with histone acetyltransferases (HATs), and pRB with histone deacetylases (HDACs) [18–20]. These enzymes are best known for their role in controlling transcription by altering chromatin structure: HAT activity promotes an 'open' chromatin configuration that allows recruitment of transcription factors that activate transcription, while HDAC activity promotes a 'closed' configuration that represses transcription [21,22]. There is good evidence that E2F and pRB family proteins regulate transcription of E2F target genes by respectively recruiting HATs and HDACs to alter chromatin structure [20,23]. A simple extension of this idea to the recruitment and formation of replication complexes might explain how pRB–E2F complexes affect gene amplification (Figure 1).

Direct binding of pRB–E2F to the ORC raises a more intriguing possibility, however. Recently it has become apparent that acetylation regulates the activity of proteins other than histones [22,24]. This group of proteins consists mostly of transcription factors, but also includes non-chromatin associated proteins. Acetylation modifies protein activity, and some have suggested that acetylation/deacetylation may rival phosphorylation as a means of controlling protein function [21,24]. Acetylation/deacetylation of certain transcription factors regulates their DNA binding ability and their interactions with other proteins. Importantly, the acetylation/deacetylation of these proteins can be carried out by the same enzymes that act on histones, for example PCAF, p300/CBP and HDAC1–4 [18,19,25]. The acetylation/deacetylation of ORC or other replication proteins mediated by E2F-containing complexes might therefore affect the ability of these factors to interact with DNA or with each other (Figure 1). Indeed, a likely histone acetyltransferase, HBO1, binds human Orc1, although whether any replication factors are modified by HBO1 is not known [26]. All of these observations suggest that perhaps the time is right to begin thinking of E2F-containing complexes not simply as transcription

Figure 1



Two non-exclusive hypotheses for how pRB–E2F complexes could directly regulate replication origin firing without modulating transcription. In the model illustrated on the left, pRB bound to E2F recruits HDAC to the chromatin, which deacetylates histones and drives the chromatin into a configuration that hinders access of replication factors to the origin. In the opposite situation, HAT activity associated with E2F causes histone acetylation and a chromatin configuration that allows the assembly of replication complexes at the origin. In an alternative model illustrated on the right, deacetylation of certain components of the replication complex (RC) by pRB–E2F-associated HDAC decreases their association with either DNA or with each other. Acetylation of these components by HAT-bound E2F increases their DNA binding ability and/or their affinity to each other, facilitating the formation of the replication complex at sites of replication initiation.

factors, but as enzymes that can affect protein function more directly.

References

1. Humbert PO, Verona R, Trimarchi JM, Rogers C, Dandapani S, Lees JA: E2f3 is critical for normal cellular proliferation. *Genes Dev* 2000, 14:690-703.
2. Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM, Rempel RE: E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Mol Cell* 2000, 6:729-735.
3. Dannenberg JH, van Rossum A, Schuijff L, te Riele H: Ablation of the Retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev* 2000, 14:3051-3064.
4. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T: Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev* 2000, 14:3037-3050.
5. Dyson N: The regulation of E2F by pRB-family proteins. *Genes Dev* 1998, 12:2245-2262.
6. Du W: Suppression of the ***rbf*** null mutants by a ***de2f1*** allele that lacks transactivation domain. *Development* 2000, 127:367-379.
7. Calvi BR, Spradling AC: Chorion gene amplification in ***Drosophila***: a model for metazoan origins of DNA replication and S-phase control. *Methods* 1999, 18:407-417.
8. Austin RJ, Orr-Weaver TL, Bell SP: ***Drosophila*** ORC specifically binds to ***ACE3***, an origin of DNA replication control element. *Genes Dev* 1999, 13:2639-2649.
9. Lu L, Zhang H, Tower J: Functionally distinct, sequence-specific replicator and origin elements are required for ***Drosophila*** chorion gene amplification. *Genes Dev* 2001, in press.
10. Takisawa H, Mimura S, Kubota Y: Eukaryotic DNA replication: from pre-replication complex to initiation complex. *Curr Opin Cell Biol* 2000, 12:690-696.
11. Royzman I, Austin RJ, Bosco G, Bell SP, Orr-Weaver TL: ORC localization in ***Drosophila*** follicle cells and the effects of mutations in dE2F and dDP. *Genes Dev* 1999, 13:827-840.
12. Bosco G, Du W, Orr-Weaver T: DNA replication control through interaction between E2F/RB and the origin recogntion complex. *Nat Cell Biol* 2001, 3:289-295.
13. Calvi BR, Lilly MA, Spradling AC: Cell cycle control of chorion gene amplification. *Genes Dev* 1998, 12:734-744.
14. Asano M, Wharton RP: E2F mediates developmental and cell cycle regulation of ORC1 in ***Drosophila***. *EMBO J* 1999, 18:2435-2448.
15. Loebel D, Huikeshoven H, Cotterill S: Localisation of the DmCdc45 DNA replication factor in the mitotic cycle and during chorion gene amplification. *Nucleic Acids Res* 2000, 28:3897-3903.
16. Kennedy BK, Barbie DA, Classon M, Dyson N, Harlow E: Nuclear organization of DNA replication in primary mammalian cells. *Genes Dev* 2000, 14:2855-2868.
17. Knudsen ES, Buckmaster C, Chen TT, Feramisco JR, Wang JY: Inhibition of DNA synthesis by RB: effects on G1/S transition and S-phase progression. *Genes Dev* 1998, 12:2278-2292.
18. Martinez-Balbas MA, Bauer UM, Nielsen SJ, Brehm A, Kouzarides T: Regulation of E2F1 activity by acetylation. *EMBO J* 2000, 19:662-671.
19. Marzio G, Wagener C, Gutierrez MI, Cartwright P, Helin K, Giacca M: E2F family members are differentially regulated by reversible acetylation. *J Biol Chem* 2000, 275:10887-10892.
20. Harbour JW, Dean DC: Chromatin remodeling and Rb activity. *Curr Opin Cell Biol* 2000, 12:685-689.
21. Cheung WL, Briggs SD, Allis CD: Acetylation and chromosomal functions. *Curr Opin Cell Biol* 2000, 12:326-333.
22. Sterner DE, Berger SL: Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev* 2000, 64:435-49.
23. Takahashi Y, Rayman JB, Dynlacht BD: Analysis of promoter binding by the E2F and pRB families ***in vivo***: distinct E2F proteins mediate activation and repression. *Genes Dev* 2000, 14:804-816.
24. Kouzarides T: Acetylation: a regulatory modification to rival phosphorylation? *EMBO J* 2000, 19:1176-1179.
25. Luo J, Su F, Chen D, Shiloh A, Gu W: Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* 2000, 408:377-381.
26. Iizuka M, Stillman B: Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. *J Biol Chem* 1999, 274:23027-23034.